Enhancement of postsynaptic GABA_A and extrasynaptic NMDA receptor-mediated responses in the barrel cortex of *Mecp2*-null mice

**Running Head:** *Mecp2* gene regulates cortical GABA_A and NMDA receptors

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ABSTRACT

Rett syndrome (RTT) is a neurodevelopmental disorder that results from mutations in the X-linked gene for methyl-CpG-binding protein 2 (MECP2). The underlying cellular mechanism for the sensory deficits in patients with RTT is largely unknown. This study used the Bird mouse model of RTT to investigate sensory thalamocortical synaptic transmission in the barrel cortex of Mecp2-null mice. Electrophysiological results showed an excitation/inhibition (E/I) imbalance, biased towards inhibition, due to an increase in efficacy of postsynaptic GABA_A receptors density rather than alterations in inhibitory network and presynaptic release properties. Enhanced inhibition impaired the transmission of tonic sensory signals from the thalamus to the somatosensory cortex. Previous morphological studies showed an upregulation of NMDA receptors in the neocortex of both RTT patients and Mecp2-null mice at early ages (Blue et al. 1999b; 2011). Although, AMPA and NMDA receptor-mediated excitatory synaptic transmission was not altered in the barrel cortex of Mecp2-null mice, extrasynaptic NMDA receptor-mediated responses increased markedly. These responses were blocked by memantine, suggesting that extrasynaptic NMDA receptors play an important role in the pathogenesis of RTT. The results suggest that enhancement of postsynaptic GABA_A and extrasynaptic NMDA receptors-mediated responses may underlie impaired somatosensation, and that pharmacological blockade of extrasynaptic NMDA receptors may have therapeutic value for RTT.

Key Words: Rett Syndrome, Mecp2 gene, Excitation/Inhibition balance, barrel cortex.
INTRODUCTION

Rett syndrome (RTT) (Rett 1966) is a developmental disorder predominantly occurring in females. It is caused by mutations in the methyl-CpG-binding protein 2 (MECP2) gene on the X chromosome (Amir et al. 1999). MeCP2 acts a transcription repressor of many genes throughout the genome (Calfa et al. 2011; Na et al. 2013). RTT syndrome manifests itself as developmental retardation and regression beginning between ages 6–18 months. Characteristics include slowed brain and head growth, stereotypical hand movements, seizures, intellectual disability, osteoporosis, respiratory and autonomic dysfunction (Hagberg 2002; Naidu et al. 1990; Neul et al. 2010). Post mortem brains of RTT patients show a disruption of the growth of axodendritic synapses (Johnston et al. 1995) and age-related biphasic changes in NMDA, AMPA, kainic acid and metabotropic glutamate and GABA receptors in various brain regions (Blue et al. 1999b; Johnston et al. 2001; 2005).

Sensory impairments in RTT patients have been reported with somatosensory evoked potential technique (Badr et al. 1987; Bader et al. 1989; Glaze, 2005; Kalmanchey, 1990; Kimura et al. 1992; Yamanouchi et al. 1993). However, the cellular mechanisms underlying sensory deficits are poorly understood.

Mouse models provide powerful approaches in revealing cellular mechanisms of developmental brain disorders. Currently there are several Mecp2 mutant mouse lines. The initial constitutive Mecp2 deletion resulted in embryonic lethality (Tate et al. 1996). Guy et al. (2001) circumvented embryonic lethality by conditional gene deletion approach (Bird model) and yielded Mecp2-null (Mecp2<sup>−/y</sup>) males. The brain of Mecp2-null mice showed age-related changes in expression of NMDA receptors (NMDARs) (Blue et al. 2011) that was similar to that observed in patients with RTT (Blue et al. 1999a; 1999b). RTT mouse models show deficits of synaptic transmission deficits that are region-specific (for reviews see: (Boggio et al. 2010; Della Sala and Pizzorusso 2014; Monteggia and Kavalali 2009;
Na and Monteggia 2011; Na et al. 2013)). For example, deletion of the \textit{Mecp2} gene leads to decreased excitatory responses in hippocampal neurons (Chao et al. 2007), layer 5 pyramidal cells in the motor cortex (Tropea et al. 2009), in the somatosensory cortex (Dani et al. 2005), layer 2/3 pyramidal cells in the motor-frontal cortex (Wood and Shepherd 2010; Wood et al. 2009), in the visual cortex (Castro et al. 2014), and the lateral geniculate nucleus (Noutel et al. 2011). In contrast, increased excitatory responses have been noted in the nucleus of the solitary tract (Kline et al. 2010), and the hippocampus CA3 region (Calfa et al. 2015). Loss of function of \textit{Mecp2} gene results in reduction of GABAergic inhibition in hippocampal CA1 pyramidal neurons (Ma et al. 2014), thalamic ventrobasal complex (Zhang et al. 2010), ventrolateral medulla (Calfa et al. 2011; Medrihan et al. 2008), and locus coeruleus (Jin et al. 2013b). However, GABAergic inhibition is enhanced in the thalamic reticular nucleus (Zhang et al. 2010), the visual cortex (Durand et al. 2012), and the hippocampus CA3 (Calfa et al. 2015). These results imply that the effect of \textit{Mecp2} gene on synaptic transmission shows region-specificity.

We studied synaptic transmission in trigeminal thalamocortical slices (Agmon and Connors 1992; Lee et al. 2005) from \textit{Mecp2}-null mice. We show that the E/I balance in layer 4 excitatory neurons of \textit{Mecp2}-null mice is biased towards inhibition, which results from an increase of postsynaptic inhibition due to enhanced postsynaptic GABA$_A$ receptor (GABA$_A$R) efficacy. While the postsynaptic excitation mediated by both AMPA receptors (AMPARs) and NMDA receptors (NMDARs) is not altered, the excitatory response mediated by extrasynaptic NMDARs is increased remarkably. Thus, deletion of \textit{Mecp2} gene leads to an increase in postsynaptic GABA$_A$R efficacy and extrasynaptic NMDAR-mediated response in the layer IV excitatory neurons.
MATERIALS AND METHODS

Animals. Breeding Mecp2 heterozygous (Mecp2\textsuperscript{+/-}) females with C57BL/6 males yields Heterozygous females (HET) and hemizygous (Mecp2\textsuperscript{-/-}) (Mecp2-null) males (Guy et al. 2001). Since the Mecp2 gene is located in the X chromosome, hemizygous males (Mecp2 -/y) are considered “null” mutations and they do not breed. All surgical procedures followed NIH guidelines and were approved by the UMB IACUC.

Brain slice preparation. Three to five week-old wildtype (WT, n=25) and Mecp2-null male mice (n=22) were anesthetized with isoflurane and decapitated. The brain was rapidly removed and immersed in cold (< 4ºC) artificial cerebrospinal fluid (ACSF, in mM: NaCl, 125; KCl, 2.5; NaH2PO4, 1.25; MgSO4, 1; NaHCO3, 25; glucose, 25; CaCl2, 2, pH=7.4) bubbled with 95% O2 and 5% CO2. Thalamocortical slices were cut (350 µm) with a vibratome (Campden 7000msz) at an angle of 50° from the mid-sagittal plane and 10° from the coronal (Agmon and Connors 1992; Lee et al. 2005). After one hour incubation in ACSF at 33°C, the slices were kept at room temperature for at least one hour. The slice containing the thalamocortical pathway was transferred into a submerged-type recording chamber (27L, Warner Ins.) and continuously perfused (>2 ml/min) with normal ACSF at room temperature.

Electrophysiology. Whole-cell-patch micropipettes were pulled horizontally in three stages from borosilicate glass (WPI, K150F-4) with a P-87 puller (Sutter Instrument Co.). The patch electrodes were backfilled with a Cs-based intracellular solution (in mM: CsMeSO\textsubscript{3}, 115; NaCl, 10; KCl, 1, MgCl\textsubscript{2}, 4; CaCl\textsubscript{2}, 1; EGTA, 11; HEPES, 20; Na\textsubscript{2}-ATP, 3; Na\textsubscript{2}-GTP, 0.5, pH=7.25, >290 mOsm) with a tip resistance of 5-9 MΩ. Layer IV excitatory neurons in the barrels not in the septa (4X/0.13 objective) were visualized with infrared light.
(IR)/Differential Interference Contrast (DIC) optics of Olympus BX51WI upright microscope (long working distance water immersion objective 40X/ 0.80 W). After forming whole-cell configuration, depolarizing current pulses were passed through the patch pipette to identify firing pattern of excitatory neurons (Agmon and Connors 1992; Feldmeyer et al. 1999; Beierlein et al. 2003) in current clamp mode. A concentric stimulating electrode (WPI, TM33CCINS) was inserted into the thalamic ventrobasal complex (VB). Electrical monophasic pulses (0.3 ms duration, 0.2 Hz, 0-500 µA) were passed through the electrode (core negative) to evoke excitatory and inhibitory postsynaptic responses in both current- and voltage-clamp modes. Paired-pulses at an interval of 200 ms were delivered to test the paired-pulse ratio (PPR) of EPSCs and IPSCs. All biological data were acquired by an InstruTECH ITC-16 interface unit and stored on a Dell DM061 computer with a PULSE (HEKA) software program.

Multiple Input Index (MII) analysis. For excitatory connections, layer IV excitatory neurons were voltage-clamped at +60 mV. In the presence of 50 µM picrotoxin (PTX), a GABA₉R antagonist, EPSCs were induced by stimulation of VB at 0.2 Hz. The stimulus intensity was gradually increased from 0 to 500 µA at steps of 10 µA as described previously (Lo et al. 2011). The peak amplitudes of EPSCs were measured and plotted against stimulus intensity. The amplitude of EPSCs enhanced in a stepwise manner following the increase in stimulus intensity. We first measured the baseline noise of recordings and calculated the standard deviation (SD) of the noise. The variation in amplitude of EPSCs was analyzed. If the amplitude of an EPSC was larger than the prior EPSC by > 3 times of SD, a "jumping step" was defined, because the fluctuation of EPSCs induced by the same stimulus intensity was always less than 3 times of noise SD. The number of “jumping steps” (Multiple Input Index, MII) provided an estimate of the lower limit number of VB neurons that innervate the recorded cortical neuron.
For inhibitory connections, layer IV excitatory neurons were voltage-clamped at 0 mV. IPSCs were induced by stimulation of VB with increasing stimulating intensity. In the same way, the MII of IPSCs was obtained.

**Spontaneous EPSCs and spontaneous and miniature IPSCs recordings.** AMPAR-mediated spontaneous EPSCs (AMPAR sEPSCs) were recorded at a holding potential of -70 mV. GABA\(_\alpha\)R-mediated spontaneous IPSCs (sIPSCs) were recorded at 0 mV. GABA\(_\alpha\)R-mediated miniature IPSCs (mIPSCs) were recorded at 0 mV in the presence of Tetrodotoxin (TTX, 1 µM). NMDAR-mediated sEPSCs (NMDAR sEPSCs) were recorded at -70 mV in Mg\(^{2+}\)-free ACSF containing 10 µM DNQX, an AMPAR antagonist. The amplitude of sEPSCs, sIPSCs and mIPSCs was measured with MiniAnalysis Software.

**Extrasynaptic NMDAR-mediated responses.** In Mg\(^{2+}\)-free ACSF with DNQX (10 µM, an AMPAR antagonist), picrotoxin (PTX 50 µM, a GABA\(_\alpha\)R antagonist) and MK-801 (5 µM, an open channel NMDAR blocker), maximal stimulation with a single pulse (0.3 ms duration, 500 µA, core negative) at 0.1 Hz of VB induced postsynaptic NMDAR-mediated EPSCs at -70 mV in layer IV excitatory neurons, which were gradually blocked. Then, stimulation with 5-10 pulses (same as single pulse) at 100 Hz made glutamate spill out to extrasynaptic sites and induce extrasynaptic NMDAR-mediated EPSC (eNMDA-EPSC, Harris and Pettit 2008). Memantine (100 µM), a specific extrasynaptic NMDAR antagonist (Wu and Johnson 2015; Xia et al. 2010) blocked the eNMDA-EPSC. The ratio of synaptic and extrasynaptic NMDARs was calculated by the maximal amplitude of single pulse-induced EPSC/eNMDAR-EPSC.

In the present study, we did not differentiate between the spiny stellate and star pyramid classes of excitatory neurons in layer IV, because, the main functional difference between the two types is reportedly in their intracortical connections (Schubert et al., 2003;
Feldmeyer, 2012). Further, in analysis of our data, we pooled responses from both classes randomly for the two genotypes of mice, and obtained significant differences.

Here it is also important to note that RTT is more prevalent in human females than in males. We used \( \text{-/y Mecp2} \) mice, namely males, because, although girls with RTT are heterozygous for the MECP2 mutation, for mice, the hemizygous male (KO-\text{Mecp2-null}) have a phenotype more similar to the human condition, especially for mechanistic studies of developmental plasticity. Heterozygous (HET) female mice eventually become symptomatic but not until they are 3-6 months of age, thus not a very useful animal model for studying developmental mechanisms. In contrast, \text{Mecp2-null} mice (i.e., \text{-/y males}) become symptomatic as early as 3 weeks of age, the age of mice that were examined in this study.
RESULTS

We performed whole-cell patch recordings from layer IV excitatory neurons in an in vitro thalamocortical slice preparation from 3-5 week old male and female WT and Mecp2-null mice. The properties of thalamocortical synaptic transmission were compared between WT and Mecp2-null mice.

Impaired temporal summation of the thalamocortical excitatory transmission in Mecp2-null mice. Layer IV excitatory neurons from WT and Mecp2-null mice were identified by their adapting train of spikes (regular spiking, (Agmon and Connors 1992; Feldmeyer et al. 1999; Beierlein et al. 2003)) during membrane depolarization (Fig. 1A and B).

Stimulation of VB induced a monosynaptic EPSP followed by a disynaptic feedforward IPSP (Feldmeyer 2012). The IPSP was reversed in between -60 mV and -80 mV (~-70 mV), indicating it was mediated by GABA$_A$Rs (Fig. 1C and D, upper vs. lower traces). At -60 mV, in the WT mice, the IPSP shortened the EPSP, but did not hyperpolarize the membrane potential below -60 mV, i.e., the amplitude of EPSP≥ IPSP (Fig. 1C upper trace). However, in Mecp2-null mice the IPSP at -60 mV always hyperpolarized the membrane potential below baseline (Fig.1D upper trace) indicating that the amplitude of the EPSP was less than the IPSP. Thus, in layer IV excitatory neurons from Mecp2-null mice, inhibitory responses were relatively larger than excitatory responses.

To investigate functional processing in the barrel cortex of Mecp2-null mice, we applied a train of 50 Hz stimuli to the VB (sub-threshold for action potentials) to mimic thalamic, slowly adapting (tonic) afferents induced by whisker deflection (Simons and Carvel 1989). In the WT mice, temporal summation of postsynaptic responses at resting potential (-60 mV) built up a depolarizing plateau (Fig. 1E). The curve of averaged temporal summation of EPSPs (Fig. 1G solid line, n=16, from 8 mice) indicated that the peaks of subsequent EPSPs (measured from peak to -60 mV) were higher than the peak of the first
EPSP. When thalamic afferents are stronger, each EPSP may trigger a sodium spike, suggesting that thalamic tonic inputs are faithfully transmitted to barrel cortex. However, in Mecp2-null mice, temporal summation led to membrane hyperpolarization (Fig. 1F). The curve of averaged peaks of subsequent EPSPs (measured from peak to -60 mV, n=7, from 4 mice, Fig. 1G, dashed line) declined towards hyperpolarization. Thus, subsequent thalamic inputs failed to induce postsynaptic spikes. The outcome of temporal summation of postsynaptic excitatory responses depends upon both presynaptic transmitter release probability (Pr) and postsynaptic response properties (Lo et al. 2013). We used a paired-pulse protocol to test the Pr of thalamic afferent terminals from WT and Mecp2-null mice. EPSCs were recorded at a holding potential of +60 mV in the presence of PTX (50 µM), a GABAAR antagonist (Fig. 1H and I). The amplitude of the second EPSC was smaller than that of the first one (paired-pulse depression, PPD) in both cases. The averaged paired-pulse ratio (PPR) of EPSCs for WT mice (0.63±0.03 (n=34, from 17 mice)) was similar to that for Mecp2-null mice (0.58±0.04 (n=15, from 8 mice); p>0.37, Fig. 1J). Therefore, the Pr of thalamocortical afferent terminals did not change in Mecp2-null mice. Thus, the failure of temporal summation of thalamic excitatory inputs in Mecp2-null mice results exclusively from the change in the Excitation/Inhibition (E/I) ratio of postsynaptic responses. The E/I ratio in the thalamocortical pathway is decreased in Mecp2-null mice. In order to quantify the change in E/I ratio, we voltage-clamped layer IV excitatory neurons to the reversal potentials of GABAAR (~-70 mV) and glutamate receptors (~0 mV). As shown in Fig. 2A, between 0 mV and -70 mV, the outward (upward) current had a linear correlation (R=0.99, p=0.001) with holding potentials and closed to zero at -70 mV (Fig. 2B). The outward current at 0 mV was completely blocked by 50 µM PTX, a GABAAR antagonist (Fig. 2C, trace 1 before and trace 2 after application of PTX). Thus, the outward current at 0 mV was a GABAAR-mediated IPSC and the inward (downward) current at -70 mV was an
AMPAR-mediated EPSC because it was completely blocked by DNQX (10 µM), an AMPAR antagonist as shown in Fig. 2D. These were true for both WT and Mecp2-null mice. Then, we recorded AMPAR-mediate EPSC and GABA<sub>A</sub>R-mediated IPSC induced by maximal stimulus in each of the neurons from both types of mice (Fig. 2E and F) and calculated the ratio of AMPA/GABA. The averaged ratio of AMPA/GABA in Mecp2-null mice (0.79±0.07 (n=7 from 4 mice)) was significantly lower than that in WT mice (1.67±0.22 (n=11, from 6 mice); p<0.001; Fig. 2G).

Excitatory and inhibitory inputs to layer IV of the barrel cortex do not change in Mecp2-null mice. There are two possibilities for the decrease in E/I ratio in Mecp2-null mice: either changing neural network and/or changing expression of postsynaptic receptors. To test whether layer IV neurons of Mecp2-null mice receive fewer excitatory synaptic connections and/or more inhibitory connections, we used the multiple input index (MII, (Lo et al. 2011)) to estimate excitatory and inhibitory connections to single cortical layer IV neurons. In the presence of 50 µM PTX, the EPSCs at +60 mV were induced by stimulation of VB with increasing intensity from 0-500 µA. The amplitude of EPSCs increased in a stepwise way (representative records in Fig. 3A and B). The number of steps gave an estimate of the minimal number of innervating VB neurons (Lo et al. 2011). The averaged MII for EPSCs in WT mice (5.05±0.28 (n=20, from 10 mice)) was similar to that in Mecp2-null mice (5.13±0.21 (n=8, from 4 mice); p>0.85; Fig. 3C). Thus, layer IV neurons in Mecp2-null mice do not receive fewer thalamic excitatory connections than neurons in WT mice. We also estimated inhibitory connections in WT and Mecp2-null mice (representative records of IPSCs at 0 mV in Fig. 3D and E). Similar to EPSCs, the mean MII of IPSCs in WT mice (4.10±0.18 (n=9, from 5 mice)) was not significantly different (p>0.82) than that in MeCP2-null mouse (4.20±0.33 (n=5, from 3 mice); Fig. 3F). Thus, the layer IV neural network is unaltered in Mecp2-null mice.
We also found that PPRs of IPSCs were similar (p>0.26) in the two types of mice. Fig. 3G and H are representative records. Mean PPRs for IPSCs (0.94±0.02 (n=9, from 5 mice)) in WT mice were not significantly different from those in Mecp2-null mice (0.92±0.01 (n=13, from 7 mice): Fig. 3J). Based on these negative results, we hypothesized that the decrease in E/I ratio in the barrel cortex of Mecp2-null mice results from changes in postsynaptic receptor efficacy.

There are no changes in the postsynaptic AMPA and NMDA receptor-mediated responses in the barrel cortex of Mecp2-null mice. AMPAR-mediated spontaneous EPSCs (AMPAR-sEPSC) were recorded at -70 mV (Fig. 4A and B). The mean amplitude of AMPAR-sEPSCs in WT mice (12.5±0.4 pA, n=386, from 5 neurons of 3 mice) was not different from Mecp2-null mice (13.3±0.2 pA, n=268, from 5 neurons of 3 mice; p>0.10; Fig. 4C). NMDAR-mediated sEPSCs (NMDAR-sEPSC) were recorded at -70 mV in Mg²⁺-free ACSF with AMPAR antagonist DNQX (Fig. 4D and E). Amplitude of NMDAR-sEPSCs in WT mice (8.05±0.10 pA, n=274, from 4 neurons of 2 mice) was similar to that in Mecp2-null mice (8.15±0.12 pA, n=306, from 5 neurons of 3 mice; p>0.55; Fig. 4F). These results indicate that the density of glutamate receptors in layer IV excitatory neurons is unaltered in Mecp2-null mice.

Enhanced response of postsynaptic GABA₁ receptors in the barrel cortex of Mecp2-null mice. We tested GABA₁R-mediated sIPSCs and mIPSCs. GABA₁R-mediated sIPSCs (sIPSC) were recorded at 0 mV (Fig. 5A and B). The amplitude of sIPSCs in Mecp2-null mice (31.1±2.4 (n=147, from 4 neurons of 2 mice)) was significantly higher (p<0.001) than in WT mice (16.1±0.5 pA (n=238, from 6 neurons of 3 mice); Fig. 5C). The shift in the cumulative probability curve of Mecp2-null mice to a higher amplitude (Fig. 5D) also illustrated the difference in the sIPSC amplitude. To avoid action potential-dependent
IPSCs, we applied TTX to block sodium spikes and recorded miniature IPSCs (mIPSCs, Fig. 5E and F). The amplitude of mIPSCs in Mecp2-null mice (23.8±0.5 pA (n=249, from 5 neurons of 3 mice)) was significantly larger (p<0.001) than that in WT mice (12.5±0.5 pA (n=207, from 5 neurons of 3 mice)); Fig. 5G and H). Note that the averaged amplitude of mIPSCs for both types of mice was smaller than those of sIPSCs. We conclude that the decrease in E/I ratio in the thalamocortical pathway in Mecp2-null mice is caused by an increase in postsynaptic GABA_A efficacy without changes in postsynaptic AMPAR and NMDAR expression.

Autoradiographic studies (Blue et al. 1999b; 2011) showed increased NMDAR expression in the cerebral cortex of young girls with RTT and in two-week old Mecp2-null mice. Based on our findings we reasoned that the increased NMDARs might be located outside the postsynaptic density, i.e., in extrasynaptic membranes.

Enhancement of extrasynaptic NMDA receptor-mediated response in barrel cortex of Mecp2-null mice
Thus, we measured the response of extrasynaptic NMDARs (eNMDAR). In this set of experiments, we examined the response induced by single shock of maximal intensity to VB when postsynaptic NMDAR-mediated responses were blocked with MK-801, an open channel NMDAR blocker. A train of stimulating pulses (the same intensity) was applied at 100 Hz to induce responses of eNMDARs (denoted by arrows in Fig. 6A and B). The responses of eNMDARs were further identified by blockade with memantine, a specific eNMDAR antagonist (Wu and Johnson 2015; Xia et al. 2010). We calculated the ratio of eNMDAR-mediated response/maximal postsynaptic NMDAR-mediated response (Normalized eNMDA-EPSC in Fig. 6C). The normalized eNMDA-EPSC in Mecp2-null mice 72.8±6.4 (n=6, from 3 mice)) was significantly (p<0.001) higher than WT mice 34.8±1.7 (n=6 ,from 3 mice). Therefore, in the barrel cortex of Mecp2-null mice, expression of eNMDARs is also upregulated.
DISCUSSION

We chose the trigeminal thalamocortical pathway to investigate the underlying mechanisms of somatosensory impairment in RTT for two major reasons. First, children with RTT show prominent somatosensory cortical processing problems and associated behaviors, such as unique oral and facial sensitivities, and aberrant responses to affective touch. Second, this pathway is well characterized both anatomically and physiologically in mice and several groups including ours have used it to assess synaptic defects in a variety of mutant mouse models (Albright et al. 2007; Iwasato et al. 2008; Li et al. 2013; Lo et al. 2013; Lu et al. 2001; 2003; 2006). The deficits in the function of this pathway caused by deletion of the Mecp2 gene are unknown. Our results show that trigeminal thalamocortical synapses in Mecp2-null mice have a dramatically lower E/I ratio than in WT mice.

A proper E/I balance is critical for keeping normal neural network activity. Imbalance of E/I ratio is proposed to be a cellular mechanism underlying many neurological and psychiatric disorders, such as autism spectrum disorder, epilepsy, schizophrenia and RTT (for reviews see (Gatto and Broadie 2010; Zhang and Sun 2011)). Our findings are mostly similar to the studies in other brain regions in Mecp2-null mice. The difference is that the change in E/I ratio in other brain regions results from decreased excitatory responses (Castro et al. 2014; Chao et al. 2007; Dani et al. 2005; Noutel et al. 2011; Tropea et al. 2009; Wood and Shepherd 2010; Wood et al. 2009). However, in the barrel cortex of Mecp2-null mice, AMPAR- and NMDAR-mediated excitation is not different from that in WT mice. The decrease in E/I ratio is mainly, if not completely, caused by an increase in postsynaptic inhibition that impairs synaptic transmission of tonic sensory signals. Our results provide another example of region-specificity of Mecp2 gene’s function. The enhanced postsynaptic inhibition was demonstrated by an increase in the amplitude of GABA\textsubscript{A}R-mediated sIPSCs and mIPSCs, suggesting that the efficacy of GABA\textsubscript{A}Rs
increases in *Mecp2*-null mice. GABA$_{\alpha}$Rs mediate most of the fast inhibitory neurotransmission in the brain. They exhibit extensive structural heterogeneity as indicated by the 21 different subunit genes that encode various GABA$_{\alpha}$R subtypes. Because of cell type-specific gene expression and differential assembly of subunits into receptor complexes, various GABA$_{\alpha}$R subtypes are expressed in different neuronal cell types. Structurally distinct receptor subtypes then exhibit different GABA sensitivity and channel functions (Fritschy and Brunig 2003; Jacob et al. 2008; Luscher and Keller, 2004; Luscher et al. 2011; Michels and Moss 2007; Vithlani et al. 2011). Because synaptically released neurotransmitters (including GABA) saturate their receptors (Clements, 1996), the strength of inhibitory synaptic currents is directly correlated with the number of synaptic GABA$_{\alpha}$Rs (Nusser et al. 1997; 1998; Otis et al. 1994). Most likely, postsynaptic GABA$_{\alpha}$R density is increased in the barrel cortex of *Mecp2*-null mice. Recently, it was reported that *Mecp2* gene may modulate GABA$_{\alpha}$R trafficking in the locus coeruleus (Jin et al. 2013a), we do not know whether a similar situation exists for the thalamocortical system.

The following possibilities for the increased GABA$_{\alpha}$R efficacy in *Mecp2*-null mice are worth noting: 1) It results from a rearrangement of the inhibitory inputs, such that a cell would have the same total number, but they are now electrically closer to the soma. This possibility needs ultrastructural evidence for confirmation. 2) It is caused by changes in the receptor phenotype via subunit shifts. Subunit composition of postsynaptic GABA$_{\alpha}$Rs in the barrel cortex of WT mice is still unknown, despite GABA$_{\alpha}$Rs contain $\alpha_{5}$-subunit in the prefrontal and visual cortices (Redrobe et al, 2012; Jang et al., 2013). Without such information, it is not possible to exclude this possibility. 3) In the barrel cortex of *Mecp2*-null mice, some endogenous GABA$_{\alpha}$R positive allosteric modulators (PAMs) that bind to benzodiazepine (BZ) site may increase GABA$_{\alpha}$R-mediated current via inducing a conformational change in GABA$_{\alpha}$R structure. An endogenous GABA$_{\alpha}$R PAM has been
reported for α3-subunit containing GABA\textsubscript{A}R of thalamic reticular nucleus (Christian et al., 2013), but not for barrel cortex.

Another finding of the present study was enhancement of eNMDARs in the barrel cortex of Mecp2-null mice. NMDARs are found both on postsynaptic sites and along the surface of neurons (i.e., extrasynaptic). eNMDARs are often associated with contacts along axons and glia. eNMDARs contain NR1, NR2A or most commonly NR2B subunits and form clusters in association with scaffolding proteins such as PSD-95 and SAP102 or adhesion proteins such as cadherins (Petralia et al. 2010). eNMDARs are activated by glutamate spillover from synapses or from ectopic glutamate release from astrocytes (Hamilton et al. 2010; Jourdain et al. 2007; Matsui et al. 2005; Petralia et al. 2005). In general, there are two separate roles for differently located NMDARs: synaptic NMDA (sNMDA) receptors activate signaling pathways leading to long term plasticity (LTP/LTD) and gene expression changes that are mediated by cAMP-response element-binding-protein (CREB) and induce pro-survival events. On the other hand, Ca\textsuperscript{2+} signaling through eNMDARs shuts off CREB activity, leads to mitochondrial dysfunction, and cell death (Frasca et al. 2011; Hardingham and Bading 2010; Hardingham et al. 2002). The localization hypothesis of NMDARs suggests that activation of sNMDARs promotes cell survival, while activation of eNMDARs promotes cell death (Hardingham et al. 2002). Despite some notable recent exceptions, the localization hypothesis is strongly supported by recent observation of elevated eNMDAR expression in various neurological disorders such as Alzheimer disease, Huntington disease, ischemia/hypoxia, traumatic brain injury and epilepsy (For review see: Parsons and Raymond 2014). There is also some evidence indicating increased concentration of glutamate in the brains and CSF of children with RTT (Hamberger et al. 1992; Horska et al. 2009; Wenk 1997). Increased eNMDARs in Mecp2-null mice suggest that eNMDARs may contribute to synaptic dysfunction in RTT.
Our results showed that the eNMDAR-mediated responses were blocked by memantine in both WT and *Mecp2*-null mice. Memantine is a relatively low-affinity, open-channel blocker with a fast off-rate. Its uncompetitive nature results in an effective blockade of chronic extrasynaptic NMDAR activity and displays minimal adverse effects (Chen and Lipton 2006; Hardingham and Bading 2010; Lipton 2006). Memantine at therapeutic concentrations preferentially blocks extrasynaptic rather than synaptic currents mediated by NMDARs (Milnerwood et al. 2010; Okamoto et al. 2009; Wu and Johnson 2015; Xia et al. 2010), thus, it is able to inhibit the prolonged influx of Ca^{2+} ions from extrasynaptic receptors (Lipton 2006) as a neuroprotective agent for treating Alzheimer’s disease (for review see: Matsunaga et al. 2015), Huntington disease (Dau et al. 2014; Levine et al. 2010; Milnerwood et al. 2010; Olivares et al. 2012; Okamoto et al. 2009), and autism spectrum disorder (Rossignol and Frye 2014). It has not been used to treat RTT clinically. *In vitro* experiments showed that memantine was capable of partially reversing the deficient synaptic plasticity caused by the loss of *Mecp2* in hippocampal slices (Chapleau and Larimore 2013; Weng et al. 2011). Our results provide further evidence for memantine blockade of enhanced eNMDARs-mediated responses in the barrel cortex of *Mecp2*-null mice, and suggest that memantine could have a potential therapeutic value for Rett syndrome. Future studies are aimed to test whether memantine can ameliorate behavioral deficits observed in *Mecp2*-null mice.

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FIGURE CAPTIONS

**Fig. 1.** Impaired temporal summation of thalamocortical excitatory transmission in *Mecp2*-null mice. *A, B:* Whole-cell recordings of layer 4 excitatory neurons (regular spiking, RS) show similar firing patterns in wild type (WT) and *Mecp2* null mice. *C, D:* Stimulation of VB induces an EPSP-IPSP sequence, in which the IPSP reversal at ~-70 mV indicates mediation by GABA_A receptors. At resting membrane potential (~-60 mV), EPSP≥IPSP in WT mice, while EPSP<IPSP in *Mecp2* null mice (upper traces). *E, F:* Temporal summation of thalamic tonic (repetitive) inputs is impaired in *Mecp2*-null brain slices. *G:* Plot averaged peak amplitudes show temporal summation curves of EPSPs for WT mice (solid line) and *Mecp2-null* mice (dash line). The error bars indicate standard error. Note that in *Mecp2*-null mice, subsequent EPSPs are much smaller than the first EPSP. The large SE error bars in Mecp2-null mice result from two factors: 1. The change in amplitudes of subsequent EPSPs in Mecp2-null mice is about 80% of the first EPSP, while that for WT mice is only 20% in the normalized temporal summation curves. 2. Sample size for Mecp2-null is 7, while that of WT mice is 16. *H, I:* Example records of paired pulse depression in WT and *Mecp2*-null mice. *J:* The averaged paired-pulse ratio, an index for presynaptic release probability, is not significantly different in *Mecp2*-null brain slices compared to the WT.

**Fig. 2.** Excitation/Inhibition ratio in the thalamocortical pathway is decreased in *Mecp2*-null mice. *A, B:* Voltage-clamping of layer 4 barrel neurons to the reversal potentials of glutamate receptors (~0 mV) and of GABA_A receptors (~-70 mV) isolates GABA_A receptor-mediated IPSC and AMPA receptor-mediated EPSC separately. *C:* GABA_A receptor-mediated IPSC is blocked by picrotoxin (PTX, trace 1 before and trace 2 after application).
D: AMPA receptor-mediated EPSC is blocked by DNQX (trace 1 before and trace 2 after application). E, F: Example records of EPSCs and IPSCs from the same neurons from WT and Mecp2-null mice. G: The ratio of Excitation/Inhibition (AMPA/GABA) is decreased in Mecp2-null slices compared to those in the WT.

Fig. 3. Excitatory and inhibitory inputs to layer IV of the barrel cortex do not change in Mecp2-null mice. A, B: Representative recordings of multiple input index (MII) for EPSCs. C: There is no significant difference in MII for EPSCs between WT and Mecp2-null mice. D, E: Example records of MII of IPSCs. F: MIIIs of IPSCs are similar in WT and Mecp2-null mice. G, H: Representative recordings of paired-pulse ratio (PPR) of IPSCs. I: PPRs of IPSCs are similar for both groups of mice.

Fig. 4. Postsynaptic AMPA and NMDA receptor responses are not altered in barrel cortex of Mecp2-null mice. A, B: Representative recordings of AMPA receptor-mediated spontaneous EPSCs (sEPSCs). C: The averaged amplitude of sEPSCs in Mecp2-null mice is similar to that in WT mice. D, E: Representative recordings of NMDA receptor-mediated sEPSCs. F: There is no difference in the amplitude of sEPSCs between WT and Mecp2-null mice.

Fig. 5. Upregulated response of postsynaptic GABA_A receptors in barrel cortex of Mecp2-null mice. A, B: Representative recordings of GABA_A receptor-mediated spontaneous IPSCs (sIPSCs). C, D: The averaged amplitude of sIPSCs is larger in Mecp2-null mice than that in WT mice. E, F: Representative recordings of GABA_A receptor-mediated miniature IPSCs (mIPSCs). G, H: The averaged amplitude of mIPSCs in Mecp2-null mice is also larger than that for WT mice.
Fig. 6. Extrasynaptic NMDA receptor-mediated response is upregulated in the barrel cortex of Mecp2-null mice. A, B: Representative recordings show extrasynaptic NMDA receptor-mediated responses. In Mg²⁺ free ACSF with AMPA and GABA receptor antagonists and MK-801, an open channel NMDA receptor blocker, VB stimulation with single pulses at 0.1 Hz induces post-synaptic NMDA receptors-mediated EPSCs (black circles) that are gradually blocked. Following that, stimulation with 5-10 pulses at 100 Hz evokes extrasynaptic NMDAR (eNMDAR)-mediated EPSCs (stars, denoted by arrows). Note that in Mecp2-null mice (B), the amplitude of eNMDAR-mediated EPSCs is higher than in WT mice (A). The eNMDAR antagonist memantine blocks eNMDAR-mediated EPSCs. C: The normalized eNMDAR-mediated EPSC is larger in Mecp2-null mice than that in WT mice.
REFERENCES


Blue ME, Kaufmann WE, Bressler J, Eyring C, O'Driscoll C, Naidu S, Johnston MV. 


Christian CA, Herbert AG, Holt RL, Peng K, Sherwood KD, Pangratz-Fuehrer S, Rudolph U, Huguenard JR. Endogenous positive allosteric modulation of


Frasca A, Aalbers M, Frigerio F, Fiordaliso F, Salio M, Gobbi M, Cagnotto A,


Johnston MV, Blue ME, Naidu S. Rett syndrome and neuronal development. *J Child*


Ma LY, Wu C, Jin Y, Gao M, Li GH, Turner D, Shen JX, Zhang SJ, Narayanan V,


Na ES, Nelson ED, Kavalali ET, Monteggia LM. The impact of MeCP2 loss- or gain-of-function on synaptic plasticity. *Neuropsychopharmacology* 38:212-219,


Olivares D, Deshpande VK, Shi Y, Lahiri DK, Greig NH, Rogers JT, Huang X. N-methyl D-aspartate (NMDA) receptor antagonists and memantine treatment for


Wood L, Gray NW, Zhou Z, Greenberg ME, Shepherd GM. Synaptic circuit abnormalities of motor-frontal layer 2/3 pyramidal neurons in an RNA interference


Figure A shows recordings from WT (Wild Type) neurons, while Figure B shows recordings from Mecp2-null neurons. Figures C and D illustrate the effect of a hyperpolarizing current (IPSP) on membrane potential at different voltages. Figures E and F depict a more intense response at higher voltage levels. Figure G presents a graph showing the normalized excitatory postsynaptic potential (EPSP) over stimulus number, with WT (closed circles) and Mecp2-null (open circles) groups compared. Figure H illustrates the effect of hyperpolarizing pulses (H.P.) at +60 mV, with Figure I showing the same for a more intense pulse (1 nA). Figure J displays a bar graph comparing the paired-pulse ratio (EPSC) between WT and Mecp2-null neurons, with no significant difference indicated (p > 0.37).